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Examination of Australian *Streptococcus suis* Isolates From Clinically Affected Pigs in a Global Context and the Genomic Characterisation of ST1 as a Predictor of Virulence

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Highlights:

- Australian strains of *Streptococcus suis* have not evolved significantly from overseas strains
- Australian ST1 strains carry a broader range of putative virulence factors than other strains
- Australian ST1 strains have significantly smaller genome sizes than other strains
- Australian clones associated with disease in pigs consist predominantly of serotypes 2, 3 and ½
- Australian strains have a high prevalence of resistance to tetracycline and erythromycin.
- A low level of resistance to penicillin (8.1%) was seen, however all isolates were susceptible to enrofloxacin

Abstract

Streptococcus suis is a major zoonotic pathogen that causes severe disease in both humans and pigs. Australia's pig herd has been quarantined for over 30 years, however *S. suis* remains a significant cause of disease. In this study, we investigated *S. suis* from 148 cases of clinical disease in pigs from 46 pig herds over a period of seven years, to determine the level of genetic difference from international isolates that may have arisen over the 30 years of separation. Isolates underwent whole genome sequencing, genome analysis and antimicrobial susceptibility testing. Data was compared at the core genome level to clinical isolates from overseas. Results demonstrated five predominant multi-locus sequence types and two major *cps* gene types (cps2 and 3). At the core genome level Australian isolates clustered predominantly within one large clade consisting of isolates from the UK, Canada and North America. A small proportion of Australian swine isolates (5%) were phylogenetically associated with south-east Asian and UK isolates, many of which were classified as causing systemic disease, and derived from cases of human and swine disease. Based on this dataset we provide a comprehensive outline of the current *S. suis* clones associated with disease in Australian pigs and their global context, with the main finding being that, despite three decades of separation, Australian *S. suis* are genomically similar to overseas strains. In

addition, we show that ST1 clones carry a constellation of putative virulence genes not present in other Australian STs.

Keywords

Streptococcus suis; ST1; Australia; genetics; antimicrobial resistance

Introduction

Streptococcus suis is an agent of serious concern for the global swine industry, and an emerging zoonotic agent causing meningitis, sepsis, arthritis, endocarditis and endopthalmitis in humans. In swine, *S. suis* is predominantly carried on the tonsils, but also within nasal cavities, genital and gastrointestinal tracts of many clinically healthy pigs, a feature which complicates management strategies in herds with disease outbreaks. Despite this carriage in healthy pigs, *S. suis* is a cause of a wide range of clinical syndromes ranging from sudden death in the peracute manifestation of disease, to meningitis, septicaemia, endocarditis, arthritis and pneumonia. The clinical manifestations generally occur in the post-weaning period, although can occur less frequently in suckers and in adult pigs (Gottschalk, 2011). Isolates obtained from areas indicative of invasive disease such as joints, brain tissue, heart and abdomen may be referred to as systemic, or causative of systemic infection, in contrast to isolates obtained from lung tissue.

Human disease is particularly prevalent in the western Pacific and south-east Asian regions, where risk is predominantly attributable to poor sanitation at slaughter and ingestion of undercooked pork, followed by Europe and then significantly less in the Americas (Huong et al., 2014). Zoonotic infection with *S. suis* has occurred in Australia, although there are few reports in the literature. The most recently published cases occurred in 2007 and 2008 in an

abattoir worker from Victoria and two piggery workers from New South Wales respectively (Kennedy et al., 2008; Tramontana et al., 2008) with each case attributed to serotype 2, which, along with serotype 14, is one of two globally-dominant serotypes detected in human cases.

Treatment for affected pigs is usually reliant on administration of β-lactams such as penicillin and amoxicillin, and, where permitted, farms may prophylactically treat all pigs at the peri-weaning stage. Another management option employed by some producers is vaccination, usually in the form of bacterins produced as autogenous vaccines from on-farm isolates (Varela et al., 2013). Due to the highly variable antigenicity of the capsular polysaccharides (CPS), *S. suis* is currently classified into 29 serotypes (six previously classified serotypes have been reassigned to different bacterial species) (Tien le et al., 2013), and it is considered that protection (if any) is only provided by homologous vaccine serotypes. In addition to this, virulence factors and yet to be determined genetic factors may play a role in poor vaccine efficacy (Segura, 2015), although virulence factors are not necessarily protective antigens and the current uncertainty surrounding the role of virulence factors makes this difficult to assess.

A recent review on the worldwide distribution and typing of *S. suis* provided a comprehensive analysis of the predominant serotypes and MLSTs in swine production systems across the US, South America, Europe and South-East Asia (Goyette-Desjardins et al., 2014). Of note in this review was the lack of information on Australian *S. suis* types, with no published surveillance since 1994 (Mwaniki et al., 1994), a gap of 20 years. Following this a study published in 2015 on 45 Australian *S. suis* isolates (encompassing 3 human isolates from 2006-2008, and 42 swine isolates from 1981-2011) described 4 MLST's (1, 25, 369 and 28) (Groves et al., 2015).

Given the paucity of data available on *S. suis* associated with disease in Australian pigs, the aim of this study was to analyse and characterise a significant number of *S. suis* isolates from

diseased pigs obtained across multiple production sites and spanning a seven year period. Australia has not imported pigs for over 30 years (Todd, 1988), and has strong regulations which exclude the use of critically important antimicrobials in Australian livestock, for example prohibiting the use of fluoroquinolones (Abraham et al., 2017). Given the level of genetic diversity in *S. suis*, it was hypothesised that Australian isolates would form a subset which has diverged significantly from those overseas. Following this hypothesis, Australian strains would be expected to have modified putative virulence genes due to independent evolution as a result of geographic isolation and potentially different targets for vaccine development. Additionally antimicrobial resistance profiles are hypothesised to differ due to acquisition of distinct antimicrobial resistance determinants with the unique selection pressures in the Australian livestock sector. In this study, we report on virulence factors and antimicrobial susceptibility, and compare the core genome to international isolates to assess evolution in a global context.

Methods

Samples

A total of 148 swabs from archived clinical isolates were transferred from ACE Laboratory Services (a major swine-industry referral lab) to the Murdoch University Antimicrobial Resistance and Infectious Diseases Laboratory for detailed molecular analysis. The majority of isolates were acquired from the lungs of diseased pigs and were classified as potentially virulent, followed by isolates from heart, brain, abdomen and joints, which were classified as virulent isolates, and miscellaneous sites including upper respiratory tract, abscesses and lymphoid tissue (Table 1). Isolates spanned a seven year period with 2, 1, 29, 27, 9, 49 and 29 isolates from years 2010, 2011, 2013, 2014, 2015, 2016 and 2017 respectively, and two isolates for which the year was unknown. Isolates were originally obtained from at least 10 of

Australia's major pig production enterprises (17 samples were of unknown origin) and encompassed 46 known individual farms (7 isolates had unavailable farm data). Of the available farm data, 39 farms accounted for between one and five isolates each, five farms accounted for between six and eight isolates each and one farm accounted for 33 isolates.

Isolates.

Swabs or fresh tissue from diseased pigs were submitted by consultant veterinarians to ACE Laboratory Service, Victoria, Australia. Samples were plated onto Sheep Blood Agar (SBA), MacConkey Agar and Chocolate Agar (Oxoid, Thermo Fisher Scientific), and incubated at 37°C for 24 hours. Suspect *S. suis* isolates, which showed alpha haemolysis on SBA and that were assessed to demonstrate morphology consistent with *S. suis* were sub-cultured onto fresh plates for isolation. Identification was carried out using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) typing (Bruker). Following confirmation, isolates were harvested from SBA plates into 1ml of Tryptic Soy Broth (TSB) +10% glycerol and stored at -80°C in sterile microcentrifuge tubes. A random subset of isolates was thawed and streaked on to SBA for resurrection at 37°C. Purity of each isolate was confirmed prior to harvesting plates onto transport swabs for postage to Murdoch University.

MIC Testing.

All isolates were subjected to antimicrobial susceptibility testing via broth microdilution according to the Clinical Laboratory Standards Institute (CLSI) Performance Standards. MIC results were categorized as susceptible, intermediate and resistant using the clinical interpretative criteria specified in CLSI performance standard VET01-S3 (CLSI, 2015a). If interpretive criteria was not present in VET01-S3, CLSI performance standard M100-S25

was used (CLSI, 2015b). *Streptococcus pneumoniae* ATCC 49619and *Staphylococcus aureus ATCC 29213* were used as a control strains as per CLSI VET01-S3.

Whole-genome sequencing.

DNA extractions were performed on the 148 isolates using a MagMax DNA multi-sample kit (ThermoFisher Scientific) according to the manufacturer's instructions, with the modification to omit the RNAse treatment step. Library preparation was performed with a Nextera XT kit with the only change from the manufacturer's instructions being an increased tagmentation time of 7 minutes. Sequencing was performed on an Illumina Nextseq 500 platform using a mid-output V2 (2 x 150 cycles) reagent kit.

Sequence analysis

All sequencing files were uploaded to the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) and parsed through the Nullarbor bioinformatics pipeline (v1.20) (Seeman et al.) to determine MLST and antimicrobial resistance genes. A database was manually created containing all capsular serotypes and 40 previously described putative virulence genes (Segura et al., 2016). The Abricate (Nullarbor) programme was used to query contig files against the database to determine capsular type and virulence genes, with cutoffs of ≥95% coverage and ≥99% identity used to determine gene presence. Distinguishing serotype 2 from serotype 1/2, and serotype 1 from serotype 14 was performed as outlined by Athey et al. (2016a). Analysis of *mrp* and *epf* variants was based on in-silico analysis of sequences as per previously published data (Silva et al., 2006; Wisselink et al., 2002).

Phylogenetic trees were constructed based on single nucleotide polymorphisms (SNPs) in the core genome. For genomic comparison against international *S. suis* isolates, 383 previously

published sequences from Vietnamese, UK, North American and Canadian isolates (Athey et al., 2016b; Weinert et al., 2015) were downloaded from NCBI and ENA. Genome annotation was performed using Prokka (v1.12) (Seemann, 2014) and outputs were processed using Roary (v3.8.0) (Page et al., 2015) for core genome determination and Gubbins (v2.2.3) (Croucher et al., 2015) for recombination removal and alignment. Manual annotation of trees was performed in iTOL (v4.2) (Letunic and Bork, 2016). Sequences for new MLST allele variations were uploaded to the *Streptococcus suis* MLST Databases site (https://pubmlst.org/ssuis/) for assignment of allele identification, and final allele combinations were then uploaded for assignment of new MLSTs. All sequence read data generated in this study was deposited in the NCBI Sequence Read Archive under accession number SRP150885.

Results

Genomic and phylogenetic analysis

Genomic analysis of the 148 isolates revealed that 110 isolates belonged to 11 previously identified MLSTs with 38 isolates belonging to one of 26 newly identified MLSTs. The most prominent MLSTs were ST 27 (27/148), ST 25 (26/148), ST 28 (11/148), ST 483 (11/148) and ST 1 (10/148). The new MLSTs assigned in this study were ST 1031 – ST 1056 inclusive.

Analysis of the capsular genes resulted in two major serotypes being detected; serotype 2 (39/148) and serotype 3 (37/148). The other main serotypes were 1/2 (11/148), 16 (8/148), 19 (8/148), 8 (7/148) and 4 (7/148). Analysis of serotype and MLST combinations showed a high proportion of isolates as serotype 2 ST25 (17.6%), serotype 3 ST27 (18.2%) and

serotype 2 ST28 (6.1%). Of serotypes where more than one isolate was present, serotype 15 (n=2), 16 (n=8), 21 (n=4) and 31 (n=5) all had previously unidentified STs.

The proportion of isolates carrying the main virulence factors *mrp*, *epf* or *sly* across all isolates was 7.4% (cps 1/2, 2, 14 and 19), 6.8% (cps 1/2, 2 and 14) and 33.1% (cps1/2, 2, 3, 4, 5, 7, 8, 11, 12, 14, 15, 16, 18, 19, 21, and 23) respectively. Only a single isolate carried both *mrp* and *sly*, while 6.8% of isolates carried both *mrp* and *epf*, and 6.8% carried *mrp*, *epf* and *sly*. The isolates carrying all three genes belonged to serotypes 1/2 (n=7), 2 (n=2) and 14 and all belonged to MLST 1, with a significant association between ST 1 and the carriage of all three genes (Fisher's exact test P-value <0.0001). Although predominantly isolated from lung, two of these isolates originated from brain and two from joint fluid and all four of these isolates were serotype 1/2. These serotype 1/2 ST1 isolates also carried more of the examined putative virulence genes with an average of 46 compared to all other isolates carrying 36, and were clustered according to putative virulence gene content (Figure 1). All isolates carrying *epf* carried the short form of *epf*, also referenced as the EF protein in Wisselink et al. (2002). All *mrp* positive isolates carried a long form of *mrp*, ranging between 1128 and 1148 bp in length

The main putative virulence genes present across all isolates included *enolase* (100%), *GAPDH* (100%), *gdh* (100%), *luxS* (100%), *pgdA* (100%), *stk* (100%), *divIVA* (99.3%), *dltA* (99.3%), *dnaJ* (99.3%), *ef-tu* (99.3%), *ldh* (99.3%), *oatA* (99.3%), *srtA* (99.3%) and *ypfJ* (99.3%). Detailed examination of the virulence gene profiles with the exclusion of common genes listed above demonstrates that the Australian isolates can be grouped in multiple clades based on putative virulence gene carriage (Figure 1). Of most significance was a single clade containing a conserved block of 32 putative virulence genes, which was the only group to contain the genes *dppIV*, *epf*, *mrp*, *sbp2*, *oppA* and *lde*. This group consisted predominantly of serotype 1/2 and serotype 2 isolates, and all are ST1.

On average, the total gene content of systemic isolates from brain and joint, was 2057, significantly lower than that from respiratory isolates which carried 2109 (Mann-Whitney *U* test p-value = 0.025). When isolates from brain, joint, heart and abdomen were analysed, the average total gene content was 2095, which was not significantly different from respiratory isolates (p-value=0.682). When the total gene content of isolates was compared to the number of putative virulence genes a trend was observed based upon ST (Figure 2a). This was most apparent in the ST1 isolates which had a significantly higher number of putative virulence genes compared to the other ST's, and the lowest average total gene content of 1918 (p-value<0.00001). These findings were mirrored by an assessment of global *S. suis* virulence genes against total gene content (Figure 2b). Most striking is the consistent clustering of ST1 isolates with a mean putative virulence gene content of 45.8 and a mean total gene content of 1936. The six non-ST1 isolates within this cluster consist of ST107 (n=1), ST105 (n=2) and ST144 (n=3) which are single locus variants (SLV) of ST1. Additionally, for the published systemic isolates from Weinert et al. (2015), from which data was analysed and MLST able to be determined using the Nullarbor pipeline, 51/108 (47.2%) were ST1, with the next highest proportion being ST28 at 17/108. When SLVs were analysed, another seven isolates were found to be variants at the *gki* locus, such that when ST1 and ST1 SLV isolates were combined they accounted for 53.7% of systemic isolates.

Phylogenetic comparison of the *S. suis* isolated from Australian pigs against a global collection of *S. suis* strains resulted in the identification of four major clades (Figure 3). The strains from this study were present in all four clades. Clade 1 was highly divergent from the other clades, and contained 20 Australian strains, with strain 74-1662 (serotype 12 ST87), an isolate from lung tissue, being the most divergent. The majority of the Australian strains (89/148) clustered in clade 2, which was comprised entirely of strains from the UK, North America and Canada. Only eight strains were present in clade 4, which was made up of

Vietnamese and UK isolates. Australian serotype 2 ST25 isolates clustered with Canadian and North American serotype 2 ST25 isolates within clade 2. To explore this relationship, further analysis of the core genome was performed on these isolates (Figure 4). While Australian isolates could be distinguished from North America and Canadian strains, the small SNP difference was notable, as aside from isolate 60-154, Canadian and Australian isolates differed by a maximum of only 100 SNPs across the core genome.

Antimicrobial susceptibility testing

All 148 isolates were subjected to micro-broth dilution to determine the MIC value against ten antibiotics belonging to eight classes (Table 2). A high proportion of the isolates were resistant to both tetracycline (99.3%) and erythromycin (83.8%). Low levels of resistance were observed for florfenicol (14.9%), penicillin G (8.1%), ampicillin (0.7%) and trimethoprim/ sulphamethoxazole (0.7%). None of the isolates were resistant to enrofloxacin. Among the *S. suis* isolates, 15.6 % were multi-drug resistant (MDR; resistance to ≥3 classes of antimicrobial) (Table 3). Commonly identified MDR phenotypes included macrolide/phenicol/tetracycline (7.4%) and β-lactam/macrolide/tetracycline (6.1%). All isolates carried resistance against at least one antimicrobial class with 73.7% resistant to two classes of antimicrobial. The most common phenotypic profile was resistance to a combination of macrolides and tetracyclines with 67.6% of isolates determined to carry this resistance. When analysed by serotype using a Fisher's exact test there was no significant association between serotype and AMR phenotype. This lack of association was also seen when systemic isolates alone were assessed.

The two dominant antimicrobial resistance genes detected in these isolates were *tetO* and *ermB*. The percentage of isolates carrying identified resistance genes for tetracyclines (95.3%) and macrolides (80.5%) support the MIC data with 96.3% and 83.8% of isolates

being resistant to tetracyclines and macrolides respectively. Other resistant genes present included *aph*(3') (n=6), *fexA* (n=1), *optrA* (n=3), *lnuB* (n=6) and *spc* (n=5).

Discussion

Streptococcus suis is a major cause of disease in pigs, with the potential for significant public health impact. In this study we present a detailed assessment of *S. suis* isolates from clinically affected Australian pigs, and compare these isolates to internationally available genome sequence data. Key findings include the virulence potential of ST1 clones, the zoonotic disease potential of Australian ST1 clones which cluster with Vietnamese isolates from cases of human disease, the potential of ST1 bacterial adhesion factors as vaccine targets and the limited evolution of Australian clones from their global seed strains.

Analysis of 148 Australian isolates determined that the majority were serotype 2 (26%) or serotype 3 (25%), followed by serotype 1/2 (7.4%). This is consistent with global *S. suis* strains isolated from cases of disease, particularly for serotypes 2 and 3 in Canada, North America and China (Goyette-Desjardins et al., 2014). While the majority of isolates were obtained from lungs, 32 isolates were obtained from brain, abdomen, heart or joint, suggesting invasive or systemic isolates. Of these, 13 were serotype 2 and six were serotype 1/2. However given this relatively small sample size of systemic isolates , and the fact the association between serotype and site of isolation was not significant when assessed by systemic isolates (Fisher's exact test p=0.07), an increased pathogenicity cannot necessarily be ascribed to these serotypes. Significantly, even within this sample set, there were 20 individual serotypes detected (Supplementary Table 1).

While ST27 and ST25 were the most prevalent MLSTs, 37 individual STs were identified, including 26 new MLSTs. This greatly expands upon previously available Australian data

which documented four STs including 1, 25, 28 and 369 (Groves et al., 2015). The reason for this significant difference in MLSTs between Groves et al. (2015) and this study is unknown, but potentially due to a number of factors. Firstly, the previous study assessed 45 isolates, this study had over 100 more, increasing the likelihood of detecting other STs. Secondly, isolates from Groves et al. (2015) were all serotype 2, whereas 20 serotypes were detected here, again increasing the chances of identifying different STs. The prevalence of isolates as serotype 2 ST25 (17.6%) and serotype 2 ST28 (6.1%) is consistent with those from North America, Canada, Germany, Spain, the United Kingdom and China, and serotype 3 ST27 (18.2%) is consistent with reports from Spain and the United Kingdom (Goyette-Desjardins et al., 2014). These findings indicate that capsular and ST combinations are stable and conserved, and it is likely that the circulating strains present in Australia are reflective of the seed stock which was imported from the United Kingdom, Canada and New Zealand (Todd, 1988).

Despite conservation of serotype/ST combinations, the use of whole genome sequencing demonstrates geographic clustering at the level of the core genome. Analysis of Australian and international isolates demonstrates four clades, although more than 80% of the isolates are present in clades 2 and 4. Clade 2 consists of isolates from the United Kingdom, Canada and Australia. It can be seen that the Australian isolates form distinct clusters, which correlate with serotype/MLST combinations. Within clades however, the SNP differences are minor between Australian and international isolates, demonstrating very little evolutionary divergence. In Clade 2, Australian serotype 2 ST25 isolates (Fig 4 red) clustered within Canadian serotype 2 ST25 isolates (Fig 4 yellow). Re-deriving the core genome for these isolates showed two populations separated by geographic origin. Despite this separation, the level of evolutionary divergence has been remarkably low over a 30 year period, with most isolates from Australia and Canada separated by less than 100 nucleotide differences across the core genome. While this data supports the hypothesis that serotype/ST combinations are

stable, it nullifies our hypothesis that Australian isolates would form divergent populations which over time become phylogenetically distinct from the original clone. Only eight Australian isolates were present in clade 4, which otherwise consisted entirely of Vietnamese and UK isolates. These Australian isolates were all serotype 1/2 ST1, aside from a single isolate which was serotype 14 ST1. This appears to indicate that the MLST of *S. suis* is a better predictor of core genome phylogeny than the serotype. The presence of these isolates and their core genome similarity to the south-east Asian isolates could be due to a number of reasons. Historically, assays did not differentiate between serotypes 2 and 1/2, with isolates being reported as serotype 2 or serotype 2 (plus 1/2) (Smith et al., 1999). For this reason it may be that these isolates are derived from European serotype 1/2 isolates which were initially labelled as serotype 2. Another scenario is that a clinically normal swine worker from south-east Asia transmitted a precursor serotype 1/2 clone from the Asian region to Australian pigs, although the epidemiology and carriage in clinically unaffected humans is not well defined (Segura, 2009). Another factor to be considered is the interaction between wild birds, feral pigs and swine production systems. It has been shown that wild birds can be infected with *S. suis*, and the potential for wild bird species to contaminate feed and water sources of pig production systems with salmonella has been shown (Devriese et al., 1994), indicating potential for a similar pathway of *S. suis* transmission (Andres-Barranco et al., 2014). In addition, feral pigs could possibly harbour divergent strains of *S. suis*. Australia's feral pig population inhabit regions frequented by wild birds, and may contract *S. suis* serotypes not commonly circulating in production herds. While the extent of feral pig interaction with commercial swine is unknown, there is evidence that feral pigs move in close proximity to some production enterprises (Pearson et al., 2014).

There are an increasing number of virulence factors termed critical to virulent strains of *S. suis*, which arguably are not critical but in various combinations may affect the virulence of

different clones (Segura et al., 2017). Historically there has been a focus on three particular virulence factors; muraminidase-released protein (*mrp*), extracellular protein factor (*epf*) and suilysin (*sly*), shown to be associated with highly virulent strains, with *cps*2 strains carrying all three genes considered the most virulent globally (Oh et al., 2017). Analysis of Australian isolates revealed that serotype 2 and 3 isolates were almost exclusively *mrp*$^-$/*epf*$^-$/*sly*$^-$. This is in agreement with early studies demonstrating that the presence of all three of these factors is not necessary for a clone to exhibit high levels of virulence (Smith et al., 1996), with 9/18 (50%) of the isolates obtained from joints or brain having this gene combination. The presence of the *mrp*$^+$/*epf*$^+$/*sly*$^+$ combination in 7/11 (63.6%) of serotype 1/2 isolates, along with 4/7 of these isolates being from joints or brain, indicates that Australian serotype 1/2 isolates may have particular virulence potential, however a larger sample set, targeted to brain and joint isolates would be required to validate this. It was notable that serotype 1/2 isolates were obtained from six separate farms and six separate production enterprises, indicating that this serotype is not confined to a single company or nucleus herd. The mrp$^+$/epf$^+$/sly$^+$ combination was also carried by all ST1 isolates, and these were consistently the isolates with the largest array of putative virulence factors. Like serotype 1/2 isolates, ST1 isolates were obtained from six farms and production enterprises. All *epf*$^+$ strains carried the short form of *epf*, considered to be the most virulent of the *epf* subtypes (Smith et al., 1993). Additionally, all *mrp* subtypes were of standard length, resulting in 6.8% of isolates classified as *epf*$^+$, *mrp*$^+$,*sly*$^+$ and 6.8% of isolates being classified *epf*$^+$, *mrp*$^+$,*sly*$^-$,a genotype which has been associated with virulence (Vecht et al., 1992).

In the case of Australian ST1 isolates, a conserved constellation of virulence genes which was not present in other MLSTs provides some insight into the virulence of ST1 clones. Due to the low number of isolates in this clade (eight of known origin of which four were systemic) a correlation between systemic isolate and presence in the clade could not be

reliably inferred. While the majority of these genes have their potential virulence characteristics only defined in vitro, there is a distinct aggregation of bacterial adhesion factors such as fibronectin binding factors *dpp IV*, *mrp*, *epf* and *sbp2* and oligopeptide binding protein *oppA* (Ge et al., 2009; Li et al., 2017; Yu et al., 2016; Zheng et al., 2018). The presence of *ide*, an IgM protease would also be involved in early phase infection and attachment to cells by cleaving IgM blocking cellular binding factors (Seele et al., 2013).

When this gene block was investigated in ST1 clones from overseas, it was also found to be highly conserved across 226 isolates, with the exception of the *oppA* gene which was absent in 21.2% of isolates. Taken together these factors suggest that both Australian and international ST1 clones have a distinct fitness advantage in terms of binding ability towards host target cells, and ST1 bacterial adhesion factors may be a promising vaccine target.

In order to determine if the potential virulence of Australian ST1 isolates could be classified by analysis of the GDH amino acid sequence, as had been reported in an overseas study, we compared of the amino acid sequence of GDH across the Australian strains (Kutz and Okwumabua, 2008). Further supporting the potential virulence, only ST1 isolates had amino acid substitutions of A, S, K and K in positions 296, 299, 305 and 330 respectively, the same combination of substitutions reported by Kutz et al. (2008) in highly virulent serotype 2 clones.

Studies have shown that virulent *S. suis* clones have smaller genome sizes combined with a larger number of virulence genes when compared with less virulent isolates (Weinert et al., 2015). While the sample size of Australian isolates from sites other than the respiratory tract was too small to confirm this, the clustering of ST1 isolates as seen in Figure 2 clearly shows a high number of virulence genes in association with the lowest total gene content of all STs examined. To see if this held true on a global scale, we mapped the same data from 443

isolates which clearly demonstrated the same pattern, inclusive of six ST1 single locus variants. This data provides further evidence that ST1 clones worldwide carry high virulence gene content in combination with a low total number of genes and that virulence is more closely associated with MLST than cps type (Kerdsin et al., 2018).

Antimicrobial resistance of Australian strains was similar to levels reported overseas with regards to tetracycline (99.3%), erythromycin (83.8 %) and trimethoprim/sulfamethoxazole (0.7%). Resistance to florfenicol was 14.9%, while all isolates were clinically susceptible to enrofloxacin, likely due to this being banned from use in food producing animals in Australia. Of concern was the observed clinical resistance to penicillin G, albeit at a relatively low level in 8.1% of isolates. This is a first line therapy for *S. suis*, and indeed the β-lactams are used in human therapy (Wertheim et al., 2009). Therefore this is an aspect of *S. suis* in Australia that must be carefully monitored from both an animal and public health point of view. There was no statistically significant association between serotype and resistance profile. This is likely due to the extended number of serotypes with low numbers of observations, greatly reducing the power of statistical tests, however while some studies have demonstrated serotype associated resistance profiles (Marie et al., 2002; Wisselink et al., 2006), others have not found a clear difference (Han et al., 2001; Soares et al., 2015).

This study has greatly increased the data available on the circulating strains of *S. suis* in the Australian pig herd. Comparative genome analysis using Australian and overseas data revealed that production of autogenous vaccine stock should not be based totally on serotype of circulating strains. In fact it is our view that it is equally important to take into account MLST, as we have demonstrated that there is a strong correlation between MLST and putative virulence gene content. Additionally, the presence of bacterial adhesion factors associated with ST1 clones which were not present in other clonal groups also presents a target for future vaccine studies, potentially in the form of multivalent subunit vaccines.

In conclusion, our findings demonstrate that Australian clones of *S. suis* associated with clinical disease in pigs have maintained a stable core genome, mirroring the international seed-stock from which they were derived. Australian clones associated with disease in pigs consist predominantly of serotypes 2, 3 and 1/2, which is consistent with reports from other pig producing countries (Goyette-Desjardins et al., 2014). Despite the limited number, the characterisation of serotype 1/2 ST1 clones is significant, as all displayed distinctive factors associated with highly virulent *S. suis*, along with grouping separately to other Australian isolates in the Vietnamese/UK clade, potentially indicating a separate source of introduction. In addition to this, these strains have only low levels of divergence from Vietnamese and UK isolates from cases of swine and human systemic disease, making this a sequence type which requires further investigation.

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Table 1 Number of isolates and site of origin used in the study

Isolation Site	Number of Isolates
Abdomen	2
Brain	12
Heart	12
Joint	6
Lung	93
Lymph node	1
Neck abscess	3
Upper respiratory tract	3
Unknown	16

Table 2 Distribution of minimum inhibitory concentrations for *S. suis.*

drug	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	ns_ci	cr_ci
Ampicillin		99.3 (147)						.7 (1)					0.7(0,3.7)	0.7(0,3.7)
Ceftriaxone		88.5 (131)	7.4 (11)	2 (3)	1.4 (2)					.7 (1)			.	.
Ciprofloxacin		37.2 (55)	37.2 (55)	22.3 (33)	3.4 (5)								.	.
Enrofloxacin	14.2 (21)	44.6 (66)	40.5 (60)	.7 (1)									0.7(0,3.7)	0(0,2.5)
Erythromycin	11.5 (17)	4.1 (6)	.7 (1)	1.4 (2)			2 (3)	1.4 (2)	79.1 (117)				84.5(77.6,89.9)	83.8(76.8,89.3)
Florfenicol				2 (3)	4.1 (6)	79.1 (117)	11.5 (17)		.7 (1)	1.4 (2)	.7 (1)	.7 (1)	93.9(88.8,97.2)	14.9(9.6,21.6)
Gentamicin					6.1 (9)	40.5 (60)	43.2 (64)	9.5 (14)				.7 (1)	.	.
PenicillinG	81.8 (121)	4.7 (7)	4.7 (7)	3.4 (5)	3.4 (5)		.7 (1)		.7 (1)				12.8(7.9,19.3)	8.1(4.3,13.7)
Tetracycline				.7 (1)		.7 (1)	1.4 (2)	2 (3)	1.4 (2)	93.9 (139)			100 (97.5,100)	99.3(96.3,100)
TMS			91.9 (136)	5.4 (8)	2 (3)		.7 (1)						8.1(4.3,13.7)	0.7(0,3.7)

Percentage of isolates classified as non-susceptible (ns ci) and/or clinically resistant (cr ci) with corresponding 95% confidence intervals for those where breakpoints are available. Shaded areas indicate the range of dilutions evaluated. Vertical bars indicate clinical breakpoint (where available). TMS; trimethoprim/sulfamethoxazole

Table 3 Phenotypic AMR profiles of *Streptococcus suis* isolated from Australian pigs.

phenotype	n	%
1: mac	1	0.7
1: tet	15	10.1
2: mac_tet	100	67.6
2: phe_tet	9	6.1
3: bla_mac_tet	9	6.1
3: mac_phe_tet	11	7.4
4: bla_fpi_mac_tet	1	0.7
4: bla_mac_phe_tet	2	1.4

Breakpoints used to classify isolates are clinical breakpoints. mac; macrolides, tet; tetracyclines, phe; phenicols, bla; beta lactams, fpi; folate pathway inhibitors.

Figure 1: Presence of putative virulence factors in all 148 isolates from this study. Blocks indicate presence of individual genes. Genes are identified by columns and isolate number, the main and serotypes found in this study are identified in rows. The putative virulent clade (based upon carriage of genes) is highlighted, and *mrp*, *epf* and *sly* genes are coloured red.

Figure 2. a) Total gene content of individual Australian isolates plotted against virulence factor content based on MLST. **b)** Total gene content of international isolates for which MLST could be determined, plotted against virulence factor content.

Figure 3. Core genome SNP phylogeny of Australian and international *S. suis* isolates. Pink labels encompass clade 1, purple labels encompass clade 2, blue labels encompass clade 3 and green labels encompass clade 4. Yellow labels indicate Australian isolates. Orange fill encompasses Canadian/Nth American Serotype 2 ST25 and within this red fill encompasses Australian serotype 2 ST25.

Figure 4. Serotype 2 ST25 isolate core genome SNP phylogeny. Red clade is Canadian/Nth American isolates, blue clade is Australian clade. Remainder are Australian divergent isolates. Scale indicates SNP differences.